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PRINCIPAL INVESTIGATOR: Miklos Sahin-Toth, M.D., Ph.D.

CONTRACTING ORGANIZATION: Trustees of Boston University
Boston, MA 02118-2340

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Miklos Sahin-Toth

E-Mail: miklos@bu.edu

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Trustees of Boston University
85 E Newton St M-921
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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The aim of our research is to generate and characterize mouse models of human hereditary pancreatitis that develop pancreatitis spontaneously or exhibit increased sensitivity to experimentally induced pancreatitis. Such models are desperately needed to study in vivo the mechanistic aspects of the trypsin-dependent pathway in pancreatitis and to begin testing therapeutic and preventive approaches. Mutations in the digestive enzyme trypsinogen cause hereditary pancreatitis in humans. Previous attempts to introduce these mutant forms of human trypsinogen into mice have failed to produce models that recapitulate the human disease. Therefore, we have used mutated mouse trypsinogens that behave similarly to their human counterpart to create genetically altered mouse strains to model human hereditary pancreatitis. Specifically, we introduced mutations in the endogenous mouse T7 cationic trypsinogen gene and obtained several new mutant strains. These newly created mouse strains will be characterized with respect to spontaneous pancreatitis and sensitivity to experimentally induced pancreatitis. These studies are expected to lead to the development of novel therapeutic and preventive approaches using the novel mouse strains as test models.

15. SUBJECT TERMS

chronic pancreatitis, acute pancreatitis, trypsinogen mutation, trypsinogen activation, trypsin, knock-in mouse model, experimental pancreatitis

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1.**INTRODUCTION**: The aim of our research is to generate and characterize mouse models of human hereditary pancreatitis that develop pancreatitis spontaneously or exhibit increased sensitivity to experimentally induced pancreatitis. Such a mouse model is desperately needed to study *in vivo* the mechanistic aspects of the trypsin-dependent pathway in pancreatitis and to begin testing therapeutic and preventive approaches. To this end, we introduce mutations in the endogenous mouse T7 cationic trypsinogen, knocked-in to the T7 locus. The newly created mouse strains are then characterized with respect to spontaneous pancreatitis and sensitivity to experimentally induced pancreatitis.

2.**KEYWORDS**: chronic pancreatitis, acute pancreatitis, trypsinogen mutation, trypsinogen activation, trypsin, knock-in mouse model, experimental pancreatitis

3.**ACCOMPLISHMENTS**:

What were the major goals of the project? Our original proposal had three specific aims.
Aim 1. Identify and biochemically characterize mutations that phenocopy the effect of human hereditary-pancreatitis associated mutations in mouse trypsinogen isoform T7.
Aim 2. Generate knock-in strains with mutated T7 trypsinogen carrying mutations in the C57BL/6 background.
Aim 3. Characterize the newly created mouse strains with respect to spontaneous pancreatitis and sensitivity to experimentally induced pancreatitis.

What was accomplished under these goals?
Significant progress has been made with respect to Aim 1 (Tasks 1 and 2) and Task 3 of Aim 2. The following goals have been accomplished:

<u>Specific Aim 1</u>. Identify and biochemically characterize mutations that phenocopy the effect of human hereditary-pancreatitis associated mutations in mouse trypsinogen isoform T7.
Under this aim, we had two major tasks in our SOW: <u>Major Task 1</u> was the design and construction of mutant forms of the T7 mouse trypsinogen gene and expression and purification of these mutant enzymes. <u>Major Task 2</u> was to analyze autoactivation of the T7 trypsinogen mutants to determine which mutants might be appropriate for the generation of knock-in mice.
THIS AIM HAS BEEN COMPLETED. ALL STATED GOALS HAVE BEEN MET.
<u>Major Task 1.</u> We have constructed five mutant forms of the T7 mouse trypsinogen designed to increase autoactivation (spontaneous conversion to active trypsin). All mutations targeted the so-called activation peptide, a short N-terminal extension on trypsinogen which becomes cleaved off during activation (Figure 1). This activating cleavage can be accelerated by mutations, as we previously seen in studies on human cationic trypsinogen. A peculiarity of the trypsinogen activation peptide is the

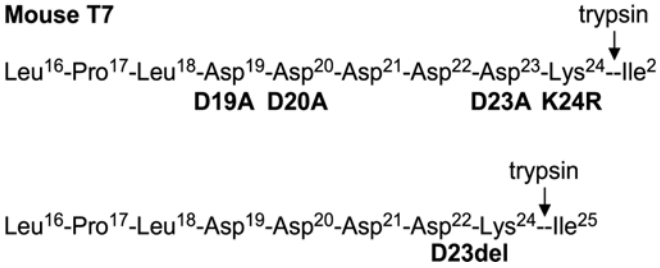

Figure 1. Mutant forms of T7 trypsinogen constructed and tested. The primary structure of the trypsinogen activation peptide in mouse T7 trypsinogen is shown. Positions mutated are indicated.

stretch of aspartic acid residues which inhibit autoactivation. Therefore, mutations were mostly designed to alter some of these residues (mutants D19A, D20A, D23A and D23del). In addition, lysine-24 was changed to arginine (mutant K24R), as trypsin cleaves after arginine faster than after lysine. Construction of mutants was accomplished using standard molecular biology methodology. Please note that amino acid numbering of trypsinogen residues in the mouse T7 trypsinogen is shifted by one relative to the human cationic trypsinogen; e.g. mutation K24R in T7 would correspond to mutation K23R in the human trypsinogen.

Major Task 2. Autoactivation of the T7 mutants was measured at pH 8.0 under various conditions; in the presence of 1 mM or 10 mM $CaCl_2$, and in the absence or presence of 150 mM NaCl. Results are shown in Figure 2 for 1 mM $CaCl_2$, and 150 mM NaCl. All mutants autoactivated faster than the wild type. Mutant D23A activated at such a rapid rate that we were unable to measure its activation under our standard assay conditions. Therefore, this mutant was placed in an inactive background containing mutation S201A which destroys the catalytic serine in trypsin. The D23A,S201A double mutant was activated by T7 trypsin and the reaction was followed by SDS-PAGE and Coomassie staining (Figure 3).

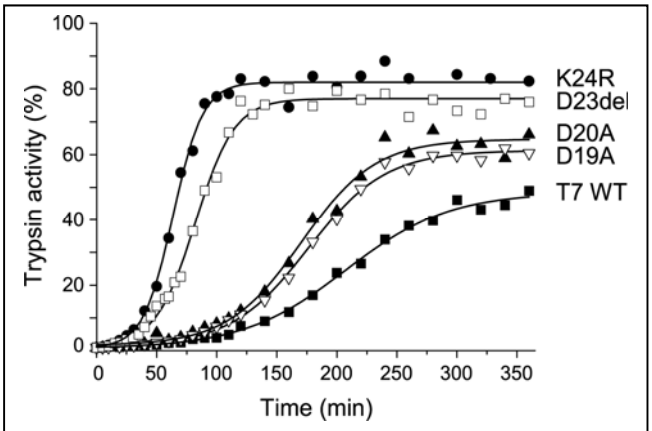

Figure 2. Autoactivation of mouse T7 trypsinogen mutants. Note that mutant D23A is shown separately in Figure 3. WT, wild type. The reaction was performed in 1 mM $CaCl_2$, 150 mM NaCl, at pH 8.0.

The outcome of these experiments was highly successful as the data show that we can choose from several mutations to induce increased autoactivation of mouse T7 trypsinogen. Most of these increases fall in the 2-4-fold range relative to wild type, whereas mutant D23A is activated about 50-fold faster! The various mutants allow us to generate multiple mouse strains, each with a given propensity for trypsinogen activation.

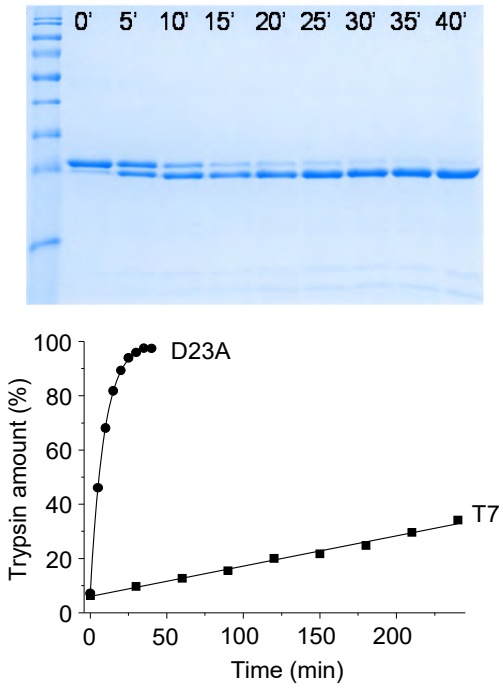

Figure 3. Activation of mouse T7 trypsinogen mutant D23A,S201A by T7 trypsin. The reaction was performed in 1 mM $CaCl_2$ and 150 mM NaCl, at pH 8.0. The representative gel picture shows the reaction for the D23A mutant. The graph demonstrates the results from the densitometric evaluation of stained gels. T7, wild-type T7 trypsinogen S201A mutant.

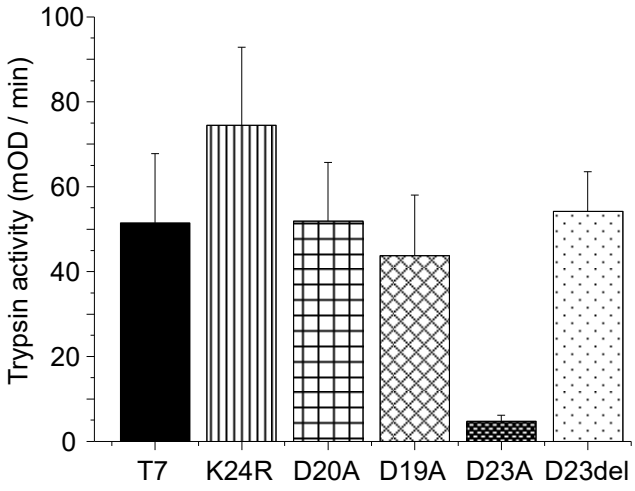

Figure 4. Cellular expression of mouse T7 trypsinogen mutants. Conditioned medium was analyzed for trypsinogen content by activation with enterokinase and measuring trypsin activity. Error bars indicate SD, n=3.

Additional experiment: Cellular expression of T7 trypsinogen mutants. We also tested how well the T7 mutants are translated and folded in the cellular environment. To this end, we cloned the mutants in the pcDNA3.1(-) expression vector and transiently transfected HEK 293T cells. After 48 hours, the conditioned medium was collected and trypsinogen secretion was determined (Figure 4). Expression of all mutants, with the exception of D23A, was comparable to that of wild-type T7. Mutant D23A was relatively poorly secreted; a phenotype we previously observed with rapidly-activating human cationic trypsinogen mutants. This experiment was important to do as it demonstrated that most mutants are likely to be expressed when knocked-in to the mouse.

SUMMARY OF RESULTS FOR SPECIFIC AIM 1. We successfully identified mutations which can phenocopy the effect of human hereditary-pancreatitis-associated mutations in the context of the mouse T7 trypsinogen. Thus, these mutations all increase autoactivation of mouse T7 trypsinogen to various extent. In the next aim, we build on these data and generate knock-in mouse strains carrying some of these mutations.

Specific Aim 2. Generate knock-in mouse strains with mutated T7 trypsinogen in the C57BL/6 background.

Under this aim, we had two major tasks in our SOW: Major Task 3 (consecutively numbered from major tasks under Specific Aim 1) was the generation of knock-in strains with mutated T7 trypsinogen and contained four sub-tasks: design and construction of targeting vectors; targeting in mouse embryonic stem cells, production of chimeric mice and production of F1 heterozygous mutant mice. Major Task 4 will be initiated, as planned in our SOW, during the second year of funding.

MAJOR TASK 3 OF THIS AIM HAS BEEN COMPLETED. ALL STATED GOALS HAVE BEEN MET.

On the basis of our results under Specific Aim 1, we expanded our original study design and selected three mutations for further analysis in the mouse: D23A, D23del and K24R. Originally, we proposed to study only D23del and K24R but the exciting *in vitro* phenotype of the D23A mutant compelled us to include this mutation as well.

To generate knock-in mice, we used the help of Cyagen, Inc., a company with significant expertise in the appropriate methodology of mouse genetics. We have worked closely with company scientists at every step of the process.

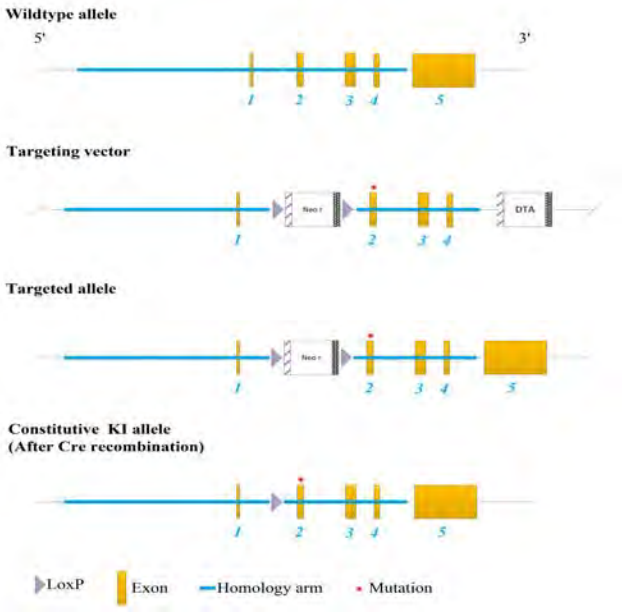

Figure 5. Targeting strategy used to generate T7 knock-in (KI) mutants. See text for details.

Targeting strategy. The T7 gene contains five exons and all mutations are targeted to exon 2 using homologous recombination in embryonic stem (ES) cells. As shown in Figure 5, the recombined allele contains a neomycin cassette (T7-Neo) to allow for positive selection of targeted ES cells. The targeting vector also contains diphtheria toxin fragment A (DTA) for negative selection. In case of specific, homologous recombination DTA is lost, whereas if non-specific integration of the targeting vector occurs the ES cells are killed by DTA. The neomycin gene is flanked by loxP sites which is used to remove the cassette by breeding with mice expressing the Cre recombinase in the mouse embryo (Cre-deleter strain). The offspring of this mating has the neomycin cassette excised in all tissues including the germline.

We expect that the presence of the neomycin cassette disrupts T7 mRNA expression and we can use these T7-Neo mice as a T7 null strain, which can serve as an additional control for our experiments. Thus, overall six strains are generated, T7-D23A-Neo, T7-D23del-Neo, T7-K24R-Neo, T7-D23A-Cre, T7-D23del-Cre and T7-K24R-Cre. The Neo designation indicates that the strain still contains the neomycin cassette, whereas the Cre designation indicates that the strain has been bred with the Cre-deleter strain and the neomycin cassette is removed. All are on the C56BL/6N substrain background. Note that even after removal of the neomycin cassette a “scar sequence” corresponding to a single loxP site is left behind in intron-1 which might potentially cause expression problems. This will be tested in the second year of funding.

Recombinant ES cells obtained

The targeting constructs were electroporated into C57BL/6N ES cells and G418-resistant clones (190 clones for D23A, 191 clones for D23del and 249 clones for K24R) were picked. Initial screening was performed by PCR using a forward primer within the Neo cassette and a reverse primer at the T7 gene intron-4/exon-5 junction. The PCR-positive clones (18 clones for D23A, 13 clones for D23del and 4 clones for K24R) were verified by DNA sequencing for the presence of the mutations, resulting in 9 D23A clones, 7 D23del clones and 4 K24R clones. Six of 9 D23A clones, 6 of 7 D23del clones and all 4 K24R clones were then expanded and verified by Southern blot analysis which confirmed all D23A and D23del clones and 2 of 4 K24R clones. The verified ES cell clones were used for injecting B6 albino (B6(Cg)-Tyrc-2J/J) blastocysts.

F1 generations of knock-in mice

Chimeras were bred against C57BL6/N and Cre-deleter strains (B6.FVBTg(EIIa-cre)C5379Lmgd/J, Jackson Laboratories) resulting in F1 generations either containing the Neo cassette or deleted for the Neo cassette (designated Cre strains as opposed to Neo strains in our experiments). After genotyping, the mice were transferred to Boston University Medical Campus where they either underwent rederivation (T7-K24R-Neo and T7-K24-Cre mice) or were quarantined and will be released soon to the barrier breeding facility. The following F1 generation mice were received at our facility:

T7-D23A-Neo, 3 males
T7-D23A-Cre (Neo-deleted), 3 males and 3 females
T7-D23del-Neo, 3 males
T7-D23del-Cre (Neo-deleted), 5 males and 3 females
T7-K24R-Neo, 4 males, 2 females
T7-K24R-Cre (Neo-deleted), 1 male and 2 females

IN SUMMARY, WE SUCCESSFULLY CREATED KNOCK-IN MOUSE STRAINS WITH MUTATED T7 TRYPSINOGEN.

What opportunities for training and professional development has the project provided?
With respect to "training", all project personnel has been continuously mentored by the PI utilizing one-on-one meetings, email exchanges and weekly group discussions. These interactions focused on the project from conceptual design to relevant technical details. With respect to "professional development", all project personnel attended departmental seminars on a regular basis (10-12 seminars in past funding year).

How were the results disseminated to communities of interest? Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?
We are exactly on track with our originally planned SOW. We will continue our research according to plan. Next, we will verify expression of the mutated T7 trypsinogens in all strains, as planned under Specific Aim 2, Major Task 4; and then move on to Specific Aim 3.

4.**IMPACT**:

What was the impact on the development of the principal discipline(s) of the project?
The project is still in its early phase. There will be a likely impact once the newly generated mouse strains are characterized in the coming years of the project. We anticipate that some of the mice will develop either spontaneous pancreatitis or will exhibit increased sensitivity to experimentally induced pancreatitis. If this were the case, we succeeded generating mouse models for hereditary pancreatitis, never before accomplished in the field of pancreatitis research. The new models will allow more detailed mechanistic studies on the role of trypsin in pancreatitis and facilitate preclinical testing of interventions designed to cure or prevent pancreatitis.

What was the impact on other disciplines? Nothing to report.
What was the impact on technology transfer? Nothing to report.
What was the impact on society beyond science and technology? Nothing to report.

5.**CHANGES/PROBLEMS**:

Changes in approach and reasons for change. On the basis of our results obtained under Specific Aim 1 we decided to generate three knock-in mouse strains (D23A, D23del and K24R) instead of the tentatively planned two. This is not a significant change in approach or scope; our experimental design remains the same; only the planned number of mice used for experiments in the future increases (no experiments have been performed on mice yet). We have an approved local IACUC amendment for this change.

In course of the completion of Specific Aim 2, we realized that the Neo-cassette containing intermediary mouse strains generated should be useful controls for our experiments. The placement of the Neo cassette in intron 1 may modify trypsinogen expression in interesting ways, e.g. may abolish it or reduce it, which can provide extra information on the relationship between phenotype (pancreatitis) and trypsinogen expression levels. Again, this does not involve a significant change in approach or scope; the experimental design remains the same, only the planned number of mice used for experiments in the future increases (no experiments have been performed on mice yet). We have an approved local IACUC amendment for this change.

The planned changes in animal numbers were also reported separately from this progress report via email to science officers Jonathan Ryder and then to Allison Milutinovich on 09-09-2015.

Actual or anticipated problems or delays and actions or plans to resolve them. No problems or delays; in fact, we have made better than expected progress.

Changes that had a significant impact on expenditures. Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

Significant changes in use or care of human subjects. No change to report.

Significant changes in use or care of vertebrate animals. The number of mouse strains investigated and consequently the total number of mice planned has increased, as described above under changes in approach. The IACUC approval date for this change is August 13, 2015.

Significant changes in use of biohazards and/or select agents. No change to report.

6.**PRODUCTS**:

Publications, conference papers, and presentations. Nothing to report.

Journal publications. Nothing to report.

Books or other non-periodical, one-time publications. Nothing to report.

Other publications, conference papers, and presentations. Nothing to report.

Website(s) or other Internet site(s). Nothing to report.

Technologies or techniques. Nothing to report

Inventions, patent applications, and/or licenses. Nothing to report

Other Products. The newly created mouse strains may prove to be important models for the study of pancreatitis, hereditary pancreatitis in particular. Additional work is needed before their utility can be fully determined.

7.PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Name: **Miklos Sahin-Toth, M.D., Ph.D.**
Project Role: PI
Researcher Identifier (e.g. ORCID ID): orcid.org/0000-0003-4513-9922
Nearest person month worked: 2 months
Contribution to Project: PI, responsible for all aspects of project.
Funding Support: This grant.

Name: **Andras Szabo, Ph.D.**
Project Role: Research Assistant Professor
Researcher Identifier (e.g. ORCID ID): orcid.org/0000-0001-8687-470X
Nearest person month worked: 6 months
Contribution to Project: Dr. Szabo worked on Specific Aims 1 and 2 of the project with a concentration on Specific Aim 1.
Funding Support: This grant.

Name: **Andrea Geisz, Ph.D.**
Project Role: Post-doctoral Associate
Researcher Identifier (e.g. ORCID ID): orcid.org/0000-0003-0971-6876
Nearest person month worked: 12 months
Contribution to Project: Dr. Geisz worked on Specific Aims 1 and 2 of the project, with a concentration on Specific Aim 2.
Funding Support: This grant.

Name: **Vera Sahin-Toth**
Project Role: Laboratory manager
Researcher Identifier (e.g. ORCID ID): None
Nearest person month worked: 6 months
Contribution to Project: Ms. Sahin-Toth provided technical support for Specific Aims 1 and 2 of the project.
Funding Support: This grant.

Name: **Laura Cosen de Binker, Ph.D.**
Project Role: Research Assistant Professor
Researcher Identifier (e.g. ORCID ID): orcid.org/0000-0003-3029-9368
Nearest person month worked: 1 month
Contribution to Project: Dr. Cosen de Binker briefly worked on the initiation of the project before leaving the PI's laboratory.
Funding Support: This grant.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? Nothing to report. No change in active support.

What other organizations were involved as partners? Nothing to report.

8.**SPECIAL REPORTING REQUIREMENTS**
COLLABORATIVE AWARDS: Not applicable.
QUAD CHARTS: Not applicable.

9.**APPENDICES**: None.